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LYMPHOTOXIN-α/ß COMPLEXES AND ANTI-LYMPHOTOXIN-BETA RECEPTOR ANTIBODIES AS ANTI-TUMOR AGENTS

TECHNICAL FIELD OF THE INVENTION

This invention relates to compositions and methods useful for activating lymphotoxin-ß receptor signaling, which in turn elicits potent anti-

5   proliferative effects on tumor cells. More particularly, this invention relates to lymphotoxin heteromeric complexes formed between lymphotoxin-α and multiple subunits of lymphotoxin-ß, which induce cytotoxic effects on tumor cells in the presence of lymphotoxin-ß receptor

10   activating agents. Also within the scope of this invention are antibodies directed against the lymphotoxin-ß receptor which act as lymphotoxin-ß receptor activating agents alone or in combination with other lymphotoxin-ß receptor activating agents either in

15   the presence or absence of lymphotoxin-α/ß complexes. A screening method for selecting such antibodies is provided. This invention also relates to compositions and methods using cross-linked anti-lymphotoxin-ß receptor antibodies in the presence of other

20   lymphotoxin-ß receptor activating agents to potentiate tumor cell cytotoxicity.

BACKGROUND OF THE INVENTION

The tumor necrosis factor (TNF) receptor family
has several members whose signaling can induce tumor cell
death by necrosis or apoptosis (programmed cell death).
5   The ligands TNF and lymphotoxin-α (LT-α; formerly called
TNF-ß) bind to and activate TNF receptors (p60 and p80;
herein called "TNF-R"). TNF-R signaling initiates
general immune responses to infection or stress in normal
cells, but is cytotoxic to cells with transformed
10   phenotypes or to tumor cells. TNF-R signaling can
selectively lyse tumor cells and virus-infected cells.
The cytotoxic effects of TNF-R signaling on tumor cells
are enhanced by interferon-γ (IFN-γ) and by a variety of
conventional chemotherapeutic agents.
15   It would be useful to take advantage of the
anti-proliferative or cytotoxic activities induced by
TNF-R signaling in tumor cells for therapeutic purposes.
However, TNF-R activation has pleiotropic effects on a
variety of immunoregulatory responses including the
20   initiation of proinflammatory cascades. Thus it has not
been possible to direct the cytotoxic effects of TNF-R
signaling to tumor cells without co-stimulating
inflammatory responses which lead to general toxicity in
humans.
25   Similarly, stimulation of another TNF-related
receptor called the Fas receptor (FasR) can trigger
cytotoxicity by programmed cell death in a variety of
both tumor and non-tumor cell types. However, FasR
activation has been shown to cause rapid liver necrosis,
30   thus precluding its therapeutic application in humans.
Recently, another receptor in the TNF family
called the LT-ß receptor (LT-ß-R) was identified (Crowe
et al., <u>Science</u>, 264, pp. 707-10 (1994)). The LT-ß-R
binds heteromeric lymphotoxin complexes (LT-α/ß) which
35   comprise LT-α subunits in association with another TNF-

related polypeptide called lymphotoxin-ß (LT-ß). These LT-α/ß complexes are membrane-associated and most have a LT-α1/ß2 stoichiometry (Browning et al., Cell, 72, pp. 847-56 (1993); Browning et al., J. Immunol., 154, pp. 33-
5   46 (1995)).

By analogy to TNF-R and other TNF-like receptors, the activation of LT-ß-R signaling is thought to occur when multiple receptors on the cell surface are brought into close proximity (Crowe et al., Science, 264,
10   pp. 707-10 (1994)). This process is referred to as receptor clustering. The TNF and LT ligands are multivalent complexes which can simultaneously bind to and thus aggregate more than one receptor. Receptor clustering as a means for receptor activation in other
15   systems has been well-documented, especially for receptor tyrosine kinases (Ullrich and Schlessinger, Cell, 61, pp. 203-212 (1990); Kolanus et al., Cell, 74, pp. 171-83 (1993)). Accordingly, administering LT-α1/ß2 ligands and/or LT-ß-R activating agents which can induce the
20   clustering and downstream signaling of LT-ß-R molecules on the surface of target tumor cells would be useful for directly stimulating the LT-ß-R pathway in these cells.

Signaling by LT-ß-R, like TNF-R, can activate pathways that lead to cytotoxicity and cell death in
25   tumor cells. Importantly, LT-α1/ß2 ligands do not bind to TNF-R with any significant affinity. For this reason, directed LT-ß-R activation in tumor cells would trigger cytotoxicity in those cells without stimulating the inflammatory pathways associated with TNF-R activation.
30   Treatment with LT-α1/ß2 and/or other LT-ß-R activating agents would thus be useful for treating or reducing the advancement, severity or effects of tumorigenic cells (neoplasia) while overcoming the potent side effect problems which have been encountered when TNF-R or FasR
35   activation has been tried as an anti-tumor treatment.

SUMMARY OF THE INVENTION

The present invention solves the problems referred to above by providing pharmaceutical compositions and methods for treating tumor cells by
5   stimulating LT-ß-R signaling without co-stimulating TNF-R-associated inflammatory responses. In one embodiment, lymphotoxin complexes formed between LT-α and multiple subunits of LT-ß are provided (LT-α/ß heteromeric complexes) which induce cytotoxic effects on cells
10   bearing the LT-ß-R in the presence of a LT-ß-R activating agent. The preferred compositions and methods of this embodiment comprise LT-α1/ß2 complexes in the presence of a LT-ß-R activating agent. More preferably, the LT-α1/ß2 complexes are in a soluble rather than a membrane-bound
15   form, and the LT-ß-R activating agent is IFN-γ.

In another embodiment of the invention, at least one antibody directed against LT-ß-R (anti-LT-ß-R Ab) is used as a second LT-ß-R activating agent in conjunction with the LT-α/ß heteromeric complex. The
20   preferred compositions and methods of this embodiment are characterized by LT-α1/ß2 in the presence of IFN-γ as a first activating agent, and at least one anti-LT-ß-R Ab as a second LT-ß-R activating agent. More preferably, the LT-α1/ß2 complexes are soluble and the antibody is a
25   monoclonal antibody (anti-LT-ß-R mAb).

In another embodiment of the invention, at least one anti-LT-ß-R Ab in the presence or absence of a second LT-ß-R activating agent is used without an exogenous LT-α/ß heteromeric complex. The preferred
30   compositions and methods of this embodiment comprise at least two anti-LT-ß-R monoclonal antibodies (anti-LT-ß-R mAbs) which recognize non-overlapping epitopes of LT-ß-R in combination with IFN-γ.

In a further embodiment, this invention
35   provides pharmaceutical compositions and methods for

potentiating tumor cell cytotoxicity characterized by cross-linked anti-LT-ß-R Abs used in conjunction with a second LT-ß-R activating agent. In one preferred embodiment, individual anti-LT-ß-R Abs are immobilized by
5 cross-linking them onto a surface. In another preferred embodiment, the anti-LT-ß-R Abs are cross-linked in solution. More preferably, the anti-LT-ß-R Abs are monoclonal antibodies and the second LT-ß-R activating agent is IFN-γ.

10 This invention further provides a novel screening process for selecting LT-ß-R activating agents, such as anti-LT-ß-R Abs, that function in combination with LT-α/ß heteromeric complexes to promote tumor cell death. The assay makes use of the increased sensitivity
15 of human adenocarcinoma cells to LT-α/ß heteromeric complexes in the presence of LT-ß-R activating agents in a cytotoxicity assay. The procedure used to test putative LT-ß-R activating agents is exemplified for the case of anti-LT-ß-R antibodies, and comprises the
20 following steps:

1)   Tumor cells (e.g., HT29 human adenocarcinoma cells) are cultured for several days in media containing IFN-γ and purified LT-α1/ß2 in the presence or absence of the particular anti-LT-ß-R Ab
25 being assayed;

2)   The cells are treated with a dye that stains living cells; and

3)   The number of stained cells is quantitated to determine the fraction of tumor cells killed in the
30 presence of the LT-α1/ß2, IFN-γ and the test anti-LT-ß-R Ab in each sample. Alternatively, the number of surviving cells can be determined by any of a number of well-known assays which measure cell viability, such as ^{3}H-thymidine incorporation into DNA.

An anti-LT-ß-R Ab (or an Ab combination) which
significantly increases the percentage of tumor cells
killed in this assay is a LT-ß-R activating agent within
the scope of this invention. This cytolytic assay can be
5   adapted to identify new LT-ß-R activating agents which
function in combination with LT-α/ß heteromeric
complexes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A. Sizing analyses of the purified LT-α/ß
10   heteromeric complex forms separated by TNF-R and
LT-ß-R immunoaffinity chromatography.   Purified proteins
were analyzed on a TSK 3000 HPLC resin in phosphate-
buffered saline.   The position of various size markers is
shown.

15   **Figure 1B.** Ion exchange analyses of purified LT forms
using a Poros carboxymethyl column (4.6mm x 100 mm) on a
BioCad instrument (Perceptive Biosystems).   27 µg of each
sample protein was loaded onto a column and eluted in a
gradient of 0 to 1M NaCl over 20 column volumes at 5
20   ml/min in a Buffer containing 16.66 µM Hepes, 16.66 µM Na
Acetate, and 16.66 µM Mes buffer (pH 6.5).

Figure 2A. Comparison of the cytotoxic activity of:
Anti-Fas receptor mAb CH-11 (-●-); TNF (-o-); LT-α (-□-);
LT-α1/ß2 (-■-); and LT-α2/ß1 (-◆-) on human
25   adenocarcinoma HT29 cells in either the presence or
absence of 80 U/ml IFN-γ.

Figure 2B. Comparison of the ability of 5 µg/ml of human
IgG (-●-), soluble p60TNF-R-Fc (-o-) and soluble
LT-ß-R-Fc receptor-immunoglobulin chimeras (-□-) to
30   inhibit the cytotoxic effects in Fig. 2A in the presence
of 80 U/ml IFN-γ.

Figure 3. Anti-LT-ß-R mAbs potentiate the cytotoxic effect of LT-α1/ß2 on human adenocarcinoma HT29 cells. **(A)** The LT-α1/ß2 cytolytic effects on HT29 cells are potentiated by the presence of anti-LT-ß-R mAb CDH10.

5   LT-α1/ß2 effects were measured without mAb (-●-), and in the presence of 0.5 µg/ml control IgG1 (-■-), 0.05 µg/ml CDH10 (-o-) and 0.5 µg/ml (-□-) CDH10. **(B)** The LT-α1/ß2 cytolytic effects on HT29 cells are inhibited by the presence of the anti-LT-ß-R mAb BDA8. LT-α1/ß2 effects

10 were measured in the presence of 2 µg/ml control IgG1 (-■-) or anti-LT-ß-R mAb BDA8 (-□-). The difference between the behavior of the CDH10 and BDA8 anti-LT-ß-R mAbs in this assay is one indication that they are directed to different epitopes of the LT-ß-R.

15 **Figure 4.** Immobilized anti-LT-ß-R mAbs are cytotoxic to human adenocarcinoma HT29 cells. **(A)** The anti-LT-ß-R mAbs have a direct cytotoxic effect on HT29 cells when they are immobilized on a surface. Plates were coated with IgG1 (-●-), a mAb directed against an unrelated

20 abundant cell surface antigen HT29/26 (-■-), BDA8 (-o-) and CDH10 (-□-). **(B)** The effects of soluble anti-LT-ß-R mAbs alone on the growth of HT29 cells. Symbols as in (A). These anti-LT-ß-R mAbs in their soluble form do not have significant cytotoxic effects on HT29 cells when

25 administered individually.

Figure 5. Representative quantitation of the enhanced cytotoxicity to tumor cells by treating with pairs of soluble anti-LT-ß-R mAbs. **(A)** The cytotoxic effects on HT29 cells of control IgG1 (100 ng/ml), anti-LT-ß-R mAb

30 BHA10 (100 ng/ml), anti-LT-ß-R mAb CBE11 (50 ng/ml), BHA10 (100 ng/ml) + IgG (100 ng/ml), and BHA10 (100 ng/ml) + CBE11 (50 ng/ml). IFN-γ was present at 80U/ml. **(B)** The cytotoxic effects on HT29 cells of control IgG1

(100 ng/ml), anti-LT-ß-R mAb CDH10 (100 ng/ml), anti-LT-ß-R mAb CBE11 (50 ng/ml), CDH10 (100 ng/ml) + IgG1 (100 ng/ml), and CDH10 (100 ng/ml) + CBE11 (50 ng/ml). IFN-γ was present at 80U/ml. **(C)** The cytotoxic effects

5 on HT29 cells of control IgG1 (100 ng/ml), anti-LT-ß-R mAb CDH10 (33 ng/ml), anti-LT-ß-R mAb AGH1 (50 ng/ml), and CDH10 (33 ng/ml) + AGH1 (50 ng/ml) on HT29 cells IFN-γ was present at 80U/ml. **(D)** As in (C), except that WiDr human adenocarcinoma cells were used in the

10 cytolytic assay (Raitano and Korc, J. Biol. Chem., 265, pp. 10466-472 (1990)).

Figure 6. Tumor size in SCID mice treated with an anti-LT-ß-R mAb. **(A)** Size of the human adenocarcinoma WiDr tumor in SCID mice 30 days after inoculation with an

15 antibody co-treatment. Mice were treated on days 1 and 2 with saline, IFN-γ alone, an anti-LT-ß-R mAb (CBE11) with and without IFN-γ and a control anti-human LFA-3 mAb (1E6) with IFN-γ. The mean of each group is indicated by a crossbar. Means, standard deviations, and number of

20 animals (in parentheticals) for the five groups (left to right) were: 0.88 +/- 0.59 (14), 1.21 +/- 0.7 (21), 0.041 +/- 0.052 (16), 0.11 +/- 0.1 (12), and 0.98 +/- 1.16 (12). **(B)** Size of the human adenocarcinoma WiDr tumor in SCID mice from 14 to 49 days after tumor cell

25 inoculation with a 15 day post-inoculation antibody treatment. Tumors were grown to an average diameter of 0.53 cm (0.076 cc) without any treatment and i.p. injections were started on day 15 and continued as indicated by the arrows. Means and standard deviations

30 are indicated for a group of 12 animals treated either with IFN-γ alone (1 x 10^6 U/injection) (-□-), IFN-γ with 50 µg 1E6 anti-LFA-3 mAb (-○-), IFN-γ with 50 µg CBE11 anti-LT-ß-R mAb (-Δ-) or 50 µg CBE11 anti-LT-ß-R mAb alone (not shown).

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be fully understood, the following detailed description is set forth.

5   The term "anti-tumor activity" refers to the ability of a substance or composition to block the proliferation of, or to induce the death of tumor cells which interact with that substance or composition.

The term "apoptosis" refers to a process of
10   programmed cell death.

The term "cytotoxic activity" refers to the ability of a substance or composition to induce the death of cells which interact with that substance or composition.

15   The term "epitope" (or antigenic determinant) is defined as the part of a molecule that combines with a single antigen binding site on an antibody molecule. A single epitope is recognized by a monoclonal antibody (mAb). Multiple epitopes are normally recognized by
20   polyclonal antibodies (Ab).

The "Fc domain" of an antibody refers to a part of the molecule comprising the CH2, CH3 and hinge regions but lacking the antigen binding sites.

The term "interferon inducing agent" refers to
25   any agent which is capable of directly or indirectly stimulating the endogenous production of either type I (IFN-α, IFN-ß) or type II (IFN-γ) interferons. Examples of interferon inducing agents include double stranded RNA molecules, and a variety of plant or pharmaceutically-
30   derived compounds.

The terms "LT-α mutein" and "LT-ß mutein" refer to LT-α or LT-ß polypeptides having one or more amino acid changes compared to the amino acid sequence of the corresponding native polypeptide.

The term "LT-ß-R activating agent" refers to any agent which can augment ligand binding to LT-ß-R, cell surface LT-ß-R clustering or LT-ß-R signaling, or which can influence how the LT-ß-R signal is interpreted
5   within the cell. Examples of LT-ß-R activating agents include IFN-α, IFN-γ, TNF, interferon inducing agents, soluble anti-LT-ß-R Abs, cross-linked anti-LT-ß-R Abs and multivalent anti-LT-ß-R Abs.

The term "LT-ß-R signaling" refers to all
10   molecular reactions associated with the LT-ß-R pathway and subsequent molecular reactions which result therefrom.

The term "anti-LT-ß-receptor antibody" ("anti-LT-ß-R Ab") refers to any antibody that recognizes and
15   binds to at least one epitope of the LT-ß receptor.

The term "anti-LT-ß receptor monoclonal antibody" ("anti-LT-ß-R mAb") refers to any monoclonal antibody that recognizes and binds to a single epitope of the LT-ß-R.

20   The term "cross-linked anti-LT-ß-R (m)Abs" refer to antibodies directed against the LT-ß-R which have either been cross-linked to each other to form antibody agglomerates in solution using an anti-LT-ß-R antibody (Ab) or (mAb) cross-linking agent, or which have
25   been immobilized in close proximity to one another on a surface or matrix.

The term "anti-LT-ß-R Ab (or mAb) cross-linking agent" refers to any agent which can covalently or non-covalently aggregate anti-LT-ß-R Abs in solution so that
30   the Abs can bind to and potentiate target cell surface LT-ß receptor clustering. Such cross-linking agents include but are not limited to chemical cross-linking agents, secondary antibodies which react with portions of the anti-LT-ß-R Abs or mAbs, and soluble or surface-bound

Fc receptors -- either endogenous or added exogenously -- which can bind to anti-LT-ß-R Abs.

The terms "LT-α biological activity", "LT-ß biological activity", and "LT-α/ß biological activity" are defined as: 1) immunological cross-reactivity with an antibody directed against at least one epitope of the corresponding native subunit or complex of subunits; or 2) the ability of the LT subunit or complex of subunits to compete for ligand binding sites on a LT-specific receptor such as TNF-R or LT-ß-R; or 3) having the ability to stimulate an immune regulatory response or cytotoxic activity qualitatively in common with a native LT subunit or complex.

The term "LT-α/ß heteromeric complex" refers to a stable association between at least one LT-α subunit and more than one LT-ß subunits. The subunits can associate through electrostatic, van der Waals, or covalent interactions. Preferably, the LT-α/ß heteromeric complex has at least two adjacent LT-ß subunits and lacks adjacent LT-α subunits. Most preferably, the complex has the stoichiometry LT-α1/ß2.

The term "multivalent ligand" refers to a molecule or complex which has more than one receptor binding site and which is capable of simultaneously binding and bringing into close proximity at least two receptor molecules.

A "type I leader sequence" is an amino-terminal portion of a eukaryotic protein which serves as a signal to direct the protein to the endoplasmic reticular (ER) membrane and often through the entire secretion pathway. The leader sequence is usually cleaved off by a signal peptidase in the ER membrane.

A "signal sequence" is the functional equivalent of a eukaryotic type I leader sequence in prokaryotic hosts, and directs the translocation of

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proteins into or across lipid bilayer membranes of a bacterium.

A "soluble LT-α/ß heteromeric complex" is a LT-α/ß heteromeric complex comprising soluble LT-ß
5   subunits, wherein the amino acid sequences which localize the polypeptide to the membrane have been deleted or inactivated, rendering the LT-ß subunit soluble. Soluble LT-α/ß heteromeric complexes can be secreted by an appropriate host cell which has been engineered to
10   express both subunits.

A "surface LT-α/ß complex" is a complex comprising LT-α and membrane-bound LT-ß subunits which is displayed on the cell surface.

Production of Membrane-bound LT-α/ß Complexes

15   Cell surface lymphotoxin complexes have been characterized in CD4^{+} T cell hybridoma cells (II-23.D7) that express high levels of LT (Browning et al., J. Immunol., 147, pp. 1230-37 (1991); Androlewicz et al., J. Biol. Chem., 267, pp. 2542-47 (1992)). Mature
20   LT-α lacks a transmembrane domain and is localized to the cell surface through interaction with at least one membrane-bound LT-ß subunit. Membrane-bound (surface) LT-α/ß heteromeric complexes have predominantly a LT-α1/ß2 stoichiometry.

25   LT-ß as a cell membrane protein binds LT-α during synthesis, thus "targeting" the LT-α to the cell membrane. In the absence of LT-ß, LT-α is secreted into the extracellular medium. LT subunits normally assemble into complexes inside the cell prior to protein export
30   into the membrane. Once LT-ß subunits are inserted into the membrane, they do not form stable complexes with secreted LT-α. Thus if the membrane-bound form of a LT-α/ß heteromeric complex is desired, it is preferable

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to co-express the desired LT-α and LT-ß subunits within the same cell.

The surface LT-α/ß heteromeric complex can be reconstructed by co-transfection of host cells with both

5   the LT-α and LT-ß genes. Surface LT complexes cannot be observed on stable cell lines which express either LT gene alone. However, if the host cell normally produces large amounts of LT-α (e.g. RPMI 1788 cells; see below), then transfection with a LT-ß gene which encodes the

10 desired LT-ß polypeptide should be sufficient to generate LT-α/ß complexes comprising full-length LT-α subunits.

Co-expression of LT-α and LT-ß polypeptides in a number of eukaryotic expression systems leads to their assembly and export as active ligand (Crowe et al., J.

15 Immunol. Methods, 168, 79-89 (1994)). Host systems that can be used include but are not limited to CHO cells, COS cells, B cells including myelomas, baculovirus-infected insect cells and yeast.

The LT-α subunit of the LT-α/ß heteromeric

20 complexes of this invention can be selected from lymphotoxin-α, native human or animal lymphotoxin-α, recombinant lymphotoxin-α, soluble lymphotoxin-α, secreted lymphotoxin-α, lymphotoxin-α muteins having LT-α biological activity, or lymphotoxin-α fragments of any of

25 the above having LT-α biological activity.

The LT-α polypeptide can be any soluble form of the molecule including active fragments thereof which can be produced in eukaryotic expression systems, wherein the natural LT-α leader sequence will be cleaved off.

30 Alternatively, fusions of the mature LT-α sequence with a heterologous signal sequence can be used to maximize the secretion of LT-α in other host systems. Signals are chosen based on the intended host cell, and may include bacterial, yeast, mammalian and viral sequences. The

35 native signal, or the vascular cell adhesion molecule-1

(VCAM-1) signal sequence is suitable for use in mammalian expression systems.

LT-α polypeptides can also be fused to polypeptides having a prolonged plasma half-life such as immunoglobulin chains or fragments thereof. Plasma proteins which may be used to enhance plasma half-life include serum albumin, immunoglobulins, apolipoproteins, and transferrin. Polyethylene glycol (PEG) attachment may stabilize the polypeptide and lower its immunogenicity. Preferably the LT-α fusion protein is not significantly immunogenic in the subject to be treated and the plasma protein does not cause undesirable side effects in subjects due to its normal biological activity.

Human LT-α is glycosylated on N and O residues, and depending on the source, exhibits considerable sugar-based microheterogeneity. The oligosaccharide composition of the particular LT-α chosen to form the LT complex may affect in vivo clearance rates (Fukushima et al., Arch. Biochem. Biophys., 304, pp. 144-53 (1993)). Since glycosylation variants can be produced by expression in different host cells, this is one factor to be considered in selecting a source of LT-α.

LT-α can be purified from a B lymphoblastoid line RPMI 1788, which constitutively secretes LT-α and which can be induced to secrete higher levels by treating with the phorbol ester PMA (Aggarwal et al., J. Biol. Chem., 259, pp. 686-91 (1984)). Alternatively, the cloned human LT-α gene can be used to recombinantly produce LT-α polypeptides in different host systems including bacteria (Schoenfeld et al., J. Biol. Chem., 266, pp. 3863-69 (1991)); baculovirus-infected insect cells (Crowe et al., J. Immunol. Methods, 168, pp. 70-89 (1994)); and mammalian cells (Browning and Ribolini, J.

Immunol., 143, pp. 1859-67 (1989); Fukushima et al., Arch. Biochem. Biophys., 304, pp. 144-53 (1993)).

Portions of the LT-α gene which encode polypeptide fragments having LT-α biological activity can

5   be evaluated using routine screening assays. Useful screening assays for LT-α biological activity include competitive inhibition assays with native LT-α bound to TNF-R, or measuring either directly or indirectly by inhibition the ability of the LT-α to induce cytotoxicity

10   of tumor cells in assays known to the art. Preferably, LT-α fragments are assembled into heteromeric complexes with LT-ß and the complexes assayed for LT-α/ß biological activity by competitive inhibition with LT-α/ß bound to LT-ß-R, or for their ability to induce cytotoxicity of

15   tumor cells in the assays disclosed herein.

Lymphotoxin-ß, also referred to as p33, has been identified on the surface of T lymphocytes, T cell lines, B cell lines and lymphokine-activated killer cells. LT-ß is the subject of applicants' co-pending

20   international applications PCT/US91/04588, published January 9, 1992 as WO 92/00329; and PCT/US93/11669, published June 23, 1994 as WO 94/13808, which are herein incorporated by reference.

The LT-ß gene encodes a polypeptide of 240-244

25   amino acids (Browning et al., Cell, 72, pp. 847-56 (1993)). LT-ß is a type II membrane protein with a short N-terminal cytoplasmic domain followed by a membrane anchoring domain of 30 hydrophobic amino acids. It has a single N-linked glycosylation site and has only one

30   cysteine residue which does not appear to be involved in intersubunit disulfide bond formation.

The LT-ß subunits comprising the LT-α/ß heteromeric complexes of the present invention can be selected from lymphotoxin-ß, native human or animal

35   lymphotoxin-ß, recombinant lymphotoxin-ß, soluble

lymphotoxin-ß, secreted lymphotoxin-ß, lymphotoxin-ß
muteins having LT-ß biological activity, or lymphotoxin-ß
fragments of any of the above having LT-ß biological
activity.

5   As discussed above for the LT-α polypeptide,
the LT-ß polypeptides can also be modified to increase
their solubility or plasma half-life using the same
methods. Likewise, portions of the LT-ß gene which
encode polypeptide fragments having LT-ß biological
10 activity can be evaluated using routine screening assays
as discussed for LT-α.

Production of soluble complexes

        Soluble (non-membrane-bound) LT-α/ß heteromeric
complexes comprise LT-ß subunits which have been changed
15 from a membrane-bound to a soluble form. These complexes
are described in detail in applicants' co-pending
international application (PCT/US93/11669, published
January 9, 1992 as WO 94/13808). Soluble LT-ß peptides
are defined by the amino acid sequence of lymphotoxin-ß
20 wherein the sequence is cleaved at any point between the
end of the transmembrane region (i.e. at about amino acid
#44) and the first TNF homology region (i.e. at amino
acid #88) according to the numbering system of Browning
et al., Cell, 72, pp. 847-56 (1993).

25   Soluble LT-ß polypeptides may be produced by
truncating the N-terminus of LT-ß to remove the
cytoplasmic tail and transmembrane region (Crowe et al.,
Science, 264, pp. 707-710 (1994)). Alternatively, the
transmembrane domain may be inactivated by deletion, or
30 by substitution of the normally hydrophobic amino acid
residues which comprise a transmembrane domain with
hydrophilic ones. In either case, a substantially
hydrophilic hydropathy profile is created which will
reduce lipid affinity and improve aqueous solubility.

Deletion of the transmembrane domain is preferred over
substitution with hydrophilic amino acid residues because
it avoids introducing potentially immunogenic epitopes.

The deleted or inactivated transmembrane domain
5   may be replaced with or attached to a type I leader
sequence (e.g. the VCAM-1 leader) such that the protein
is secreted beginning with a sequence anywhere from
between val40 to pro88. Soluble LT-ß polypeptides may
include any number of well-known leader sequences at the
10   N-terminus. Such a sequence would allow the peptides to
be expressed and targeted to the secretion pathway in a
eukaryotic system. See, e.g., Ernst et al., United
States Patent No. 5,082,783 (1992).

Soluble LT-α/ß heteromeric complexes may be
15   produced by co-transfecting a suitable host cell with DNA
encoding LT-α and soluble LT-ß (Crowe et al., J. Immunol.
Methods, 168, pp. 79-89 (1994)). Soluble LT-ß secreted
in the absence of LT-α is highly oligomerized. However,
when co-expressed with LT-α, a 70 kDa trimeric-like
20   structure is formed which contains both proteins. It is
also possible to produce soluble LT-α1/ß2 heteromeric
complexes by transfecting a cell line which normally
expresses only LT-α (such as the RPMI 1788 cells
discussed above) with a gene encoding a soluble LT-ß
25   polypeptide.

LT-α and LT-ß polypeptides may be separately
synthesized, denatured using mild detergents, mixed
together and renatured by removing the detergent to form
mixed LT heteromeric complexes which can be separated
30   (see below).

Purification of LT-α1/ß2 Complexes

Soluble LT-α1/ß2 heteromeric complexes are
separated from co-expression complexes comprising a
different subunit stoichiometry by chromatography using

TNF and LT-ß receptors as affinity purification reagents.
The TNF receptors only bind within α/α clefts of LT
complexes.   The LT-ß receptor binds with high affinity to
ß/ß clefts, and with lower affinity to α/ß clefts of

5   heteromeric LT-α/ß complexes.   Accordingly, LT-α3 and
LT-α2/ß1 will bind to TNF-R.   The LT-ß-R can also bind
LT-α2/ß1 trimers (within the α/ß clefts) but cannot bind
LT-α3.   In addition, the LT-ß-R (but not TNF-R) binds
LT-α1/ß2 and LT-ßn (the exact composition of such

10   preparation is unknown, however, they are large
aggregates).

The receptor affinity reagents can be prepared
as either a soluble extracellular domain (see for example
Loetscher et al., J. Biol. Chem., 266, pp. 18324-29

15   (1991)), or as chimeric proteins with the extracellular
ligand binding domain coupled to an immunoglobulin Fc
domain (Loetscher et al., J. Biol. Chem., 266, pp. 18324-
29 (1991); Crowe et al., Science, 264, pp. 707-710
(1994)).   Receptors are coupled to affinity matrices by

20   chemical cross-linking using routine procedures.

There are two schemes by which the LT-α1/ß2
ligand can be purified using receptors and immuno-
affinity chromatography.   In the first scheme, a
supernatant from an appropriate expression system co-

25   expressing both LT-α and the truncated LT-ß form is
passed over a TNF-R column.   The TNF-R will bind LT-α3
and LT-α2/ß1 trimers.   The flow through from the TNF-R
column will contain LT-ß(n) and LT-α1/ß2.

In the second scheme, all LT-ß-containing forms

30   (LT-ß(n), LT-α1/ß2 and LT-α2/ß1) are bound to and eluted
from a LT-ß-R column using classical methods such as
chaotrophe or pH change.   (LT-α3 flows through this
column).   The eluate is neutralized or the chaotrophe
removed, and the eluate is then passed over a TNF-R

35   column, which binds only to the LT-α2/ß1 trimers.   The

flow through of this column will contain LT-ß(n) and LT-α1/ß2 trimers.

In both cases, pure LT-α1/ß2 trimers can be separated from LT-ß by subsequent gel filtration and/or

5   ion exchange chromatographic procedures known to the art.

Alternatively, different forms of LT-α/ß heteromeric complexes can be separated and purified by a variety of conventional chromatographic means. It may also be preferable to combine a series of conventional

10 purification schemes with one of the immunoaffinity purification steps described above.

Source of Anti-LT-ß-R Antibodies

Polyclonal antibody sera directed against the human LT-ß receptor are prepared using conventional

15 techniques by injecting animals such as goats, rabbits or mice subcutaneously with a human LT-ß receptor-Fc fusion protein (Example 2) in complete Freund's adjuvant, followed by booster intraperitoneal or subcutaneous injection in complete Freunds. Polyclonal antisera

20 containing the desired antibodies which are directed against the LT-ß receptor are screened by conventional procedures.

Mouse monoclonal antibodies (mAbs) directed against a human LT-ß receptor-Fc fusion protein are

25 prepared by intraperitoneal immunization of RBF mice repetitively with a CHO cell-derived recombinant LT-ß receptor-Fc fusion protein (LT-ß-R-Fc) attached to protein A sepharose beads in the absence of adjuvant. Animals are finally boosted with soluble LT-ß-R-Fc (both

30 i.p. and i.v.), spleen cells are fused using classical protocols, and hybridomas are screened by ELISA (Ling et al., _J. Interferon and Cytokine Res._, 15, pp. 53-59 (1995)). Hybridomas are further screened for their ability to block binding of activated II-23 hybridoma

cells -- which express surface LT-α1/ß2 -- to LT-ß-R-Fc-coated plates in a cell panning assay. Pure mAbs are prepared by protein A sepharose purification of IgG from hybridoma culture supernatants.

5   Various forms of anti-LT-ß-R antibodies can also be made using standard recombinant DNA techniques (Winter and Milstein, Nature, 349, pp. 293-99 (1991)). For example, "chimeric" antibodies can be constructed in which the antigen binding domain from an animal antibody

10   is linked to a human constant domain (e.g. Cabilly et al., US 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. U.S.A., 81, pp. 6851-55 (1984)). Chimeric antibodies reduce the observed immunogenic responses elicited by animal antibodies when used in human clinical

15   treatments.

          In addition, recombinant "humanized antibodies" which recognize the LT-ß-R can be synthesized. Humanized antibodies are chimeras comprising mostly human IgG sequences into which the regions responsible for specific

20   antigen-binding have been inserted (e.g. WO 94/04679). Animals are immunized with the desired antigen, the corresponding antibodies are isolated, and the portion of the variable region sequences responsible for specific antigen binding are removed. The animal-derived antigen

25   binding regions are then cloned into the appropriate position of human antibody genes in which the antigen binding regions have been deleted. Humanized antibodies minimize the use of heterologous (inter-species) sequences in human antibodies, and are less likely to

30   elicit immune responses in the treated subject.

          Construction of different classes of recombinant anti-LT-ß-R antibodies can also be accomplished by making chimeric or humanized antibodies comprising the anti-LT-ß-R variable domains and human

35   constant domains (CH1, CH2, CH3) isolated from different

classes of immunoglobulins. For example, anti-LT-ß-R IgM
antibodies with increased antigen binding site valencies
can be recombinantly produced by cloning the antigen
binding site into vectors carrying the human μ chain

5   constant regions (Arulanandam et al., J. Exp. Med., 177,
pp. 1439-50 (1993); Lane et al., Eur. J. Immunol., 22,
pp. 2573-78 (1993); Traunecker et al., Nature, 339, pp.
68-70 (1989)).

In addition, standard recombinant DNA
10   techniques can be used to alter the binding affinities of
recombinant antibodies with their antigens by altering
amino acid residues in the vicinity of the antigen
binding sites. The antigen binding affinity of a
humanized antibody can be increased by mutagenesis based
15   on molecular modeling (Queen et al., Proc. Natl. Acad.
Sci. U.S.A., 86, pp. 10029-33 (1989); WO 94/04679).

It may be desirable to increase or to decrease
the affinity of anti-LT-ß-R Abs for the LT-ß-R depending
on the targeted tissue type or the particular treatment
20   schedule envisioned. For example, it may be advantageous
to treat a patient with constant levels of anti-LT-ß-R
Abs with reduced ability to signal through the LT-ß
pathway for semi-prophylactic treatments. Likewise,
anti-LT-ß-R Abs with increased affinity for the LT-ß-R
25   may be advantageous for short-term, tumor- targeted
treatments.

**Screening Anti-LT-ß-R Antibodies For LT-ß-R Activating
Agents**

The anti-LT-ß-R antibodies of this invention
30   can potentiate the anti-tumor activity of LT-α/ß
heteromeric complexes (preferably LT-α1/ß2) in the
presence of an LT-ß-R activating agent such as IFN-γ.
These anti-LT-ß-R antibodies are also referred to herein

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as LT-ß-R activating agents. The antibodies which act as
LT-ß-R activating agents are selected as follows:
      1)   A series of tissue culture wells
containing tumor cells such as HT29 cells are cultured
5   for three to four days in media containing a LT-ß-R
activating agent such as IFN-γ, and purified LT-α/ß
heteromeric complex -- preferably LT-α1/ß2 -- in the
presence or absence of serial dilutions of the anti-
LT-ß-R Ab being tested;
10   2)   A vital dye stain which measures
mitochondrial function such as MTT is added to the cell
mixture and reacted for several hours;
      3)   The optical density of the mixture in each
well is quantitated at 550 nm wavelength light (OD 550).
15   The OD 550 is inversely proportional to the number of
tumor cells killed in the presence of the LT-α/ß
heteromeric complex, the LT-ß-R activating agent and test
anti-LT-ß-R Ab in each well.
      The preferred antibodies of this invention
20   which act individually as LT-ß-R activating agents in the
presence of LT-α1/ß2 and IFN-γ include the BKA11, CDH10,
BHA10 and BCG6 anti-LT-ß-R mAbs (Table 2, infra).

Cross-linking Anti-LT-ß-R Antibodies
      The cross-linked anti-LT-ß-R antibodies of this
25   invention act individually as LT-ß-R activating agents
without exogenous LT-α/ß heteromeric complexes in the
presence of a second LT-ß-R activating agent such as
IFN-γ. Cross-linked anti-LT-ß-R Abs apparently bind to
and induce clustering of cell surface LT-ß receptors
30   thereby activating LT-ß receptor-mediated targeted cell
death.
      In one embodiment, one or more types of anti-
LT-ß-R antibodies are cross-linked by immobilization onto
a water-insoluble matrix or surface. Derivatization with

a bifunctional agent is useful for cross-linking the
antibodies to the water-insoluble support matrix or
surface. Agents commonly used to effect cross-linking of
antibodies to a water insoluble support matrix or surface
5   include 1,1 bis(-diazoacetyl)-2-phenylethane,
glutyraldehyde, N-hydroxysuccinamide esters including
esters with 4-azidosalicylic acid, homobifunctional
imidoesters including disuccinimidyl esters, and
bifunctional maleimides such as bis-N-maleimido-1,8-
10   octane. Derivatizing agents such as methyl-3-[(p-
azidophenyl) dithio] propioimidate form photoactivatable
intermediates which can be selectively cross-linked when
stimulated with light. Reactive water-insoluble matrices
such as cyanogen bromide-activated carbohydrates and the
15   substrates described in United States Patent Nos.
3,959,080; 3,969,287; 3,691,016; 4,195,128; 4,247,642;
4,229,537; 4,055,635; and 4,330,440 can also be used for
protein immobilization and cross-linking.

The surfaces to which the antibodies are
20   attached can be non-proteinaceous polymer, usually a
hydrophilic polymer either from natural or synthetic
sources. Hydrophilic polyvinyl polymers such as
polyvinylalcohol (PVA) and polyvinylpyrrolidone (PVP) can
be used. Also useful are polyalkylene ethers such as
25   polyethylene glycol, polypropylene glycol,
polyoxyethylene esters or methoxy polyethylene glycol,
polyoxyalkylenes such as polyoxyethylene and
polyoxypropylene, and block copolymers of polyoxyethylene
and polyoxypropylene (Pluronics); polymethacrylates;
30   carbomers; branched or unbranched polysaccharides which
comprise the saccharide monomers D-mannose, D- and L-
galactose, fucose, fructose, D-xylose, L-arabinose, D-
glucuronic acid, sialic acid, D-galacturonic acid,
D-mannuronic acid (e.g. poly-mannuronic acid or alginic
35   acid), D-glucosamine, D-galactosamine, D-glucose and

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neuraminic acid including homopolysaccharides and
heteropolysaccharides such as lactose, amylopectin,
starch, hydroxyethyl starch, amylose, dextran sulfate,
dextran, dextrins, glycogen, or the polysaccharide

5   subunit of acid mucopoly-saccharides, e.g. hyaluronic
acid; polymers of sugar alcohols such as polysorbitol and
polymannitol; and heparin or heparon.

The polymer prior to cross-linking is
preferably water soluble and preferably contains only a

10   singly reactive chemical group to avoid multiple cross-
linking events with the antibody. In any case, reaction
conditions should be optimized to reduce cross-linking
and to recover products -- either directly or by a
subsequent gel filtration or chromatographic step --

15   having a substantially homogenous molecular weight range.
The optimal molecular weight of the cross-linked antibody
matrix will be determined by routine experimentation
using the cytotoxicity and receptor binding assays
disclosed herein.

20   The final conjugate after cross-linking is
preferably soluble in physiological fluids such as blood.
The polymer should not be highly immunogenic in the
conjugate form, and should possess a viscosity compatible
with intravenous infusion or injection if either is an

25   intended route of administration.

The polymer may also be water insoluble.
Materials which may be used include hydrophilic gels, or
shaped articles having surfaces to which the antibodies
can be immobilized such as surgical tubing, catheters, or

30   drainage conduits. It is preferable to use solid support
materials which are biologically compatible and
substantially inert in physiological surroundings. A
material is biologically compatible if it does not
substantially stimulate immune responses including

inflammation, or attract fibrotic cells when placed inside the body of a subject.

Anti-LT-ß-R Abs may also be immobilized onto surfaces which have been covalently or non-covalently
5   coated with secondary antibodies that will bind to the primary anti-LT-ß-R Abs (e.g., goat anti-mouse IgG antibodies; see Example 7). Each anti-LT-ß-R mAb tested individually, when immobilized onto a surface with secondary antibodies, acts as an LT-ß-R activating agent
10   in the presence of IFN-γ (**Figures 4 and 7**).

In an alternative embodiment, cross-linked anti-LT-ß-R Abs in solution act as LT-ß-R activating agents. Anti-LT-ß-R Abs can be cross-linked by means of an anti-LT-ß-R Ab (or mAb) cross-linking agent. An
15   anti-LT-ß-R Ab (or mAb) cross-linking agent according to this invention is any agent capable of either covalently linking, or of non-covalently aggregating the anti-LT-ß-R Abs (or mAbs) in solution so that the cross-linked anti-LT-ß-R Abs (or mAbs) can bind to and potentiate target
20   cell surface LT-ß-R clustering. Such anti-LT-ß-R Ab (or mAb) cross-linking agents include but are not limited to chemical cross-linking reagents which can be reacted with the antibodies in a controlled manner as described above. Alternatively, secondary antibodies, Sepharose A, Fc
25   receptors, or other agents that will bind to and aggregate multiple primary anti-LT-ß-R Abs without blocking their activity can be used to form anti-LT-ß-R Abs agglomerates in solution.

Multiple Anti-LT-ß-R Abs In Solution Act As LT-ß-R
30   **Activating Agents**
Compositions comprising multiple anti-LT-ß-R Abs in solution which act as LT-ß-R activating agents by potentiating surface LT-ß-R clustering are provided by this invention. Polyclonal anti-LT-ß-R Abs directed
35   against different epitopes of the LT-ß-R can be used.

Preferably, the anti-LT-ß-R Abs are monoclonal Abs
directed against different and non-overlapping epitopes
of the LT-ß-R.

The combined anti-LT-ß-R mAb approach to LT-ß
5   receptor activation requires combining two non-
overlapping epitopes. Moreover, it is likely that
productive receptor aggregation is only achieved with
certain epitopes. We have identified the presence of at
least four unique LT-ß-R immunoreactive epitopes.
10   Additional epitopes (as defined by new mAbs) may be
identified by continuing to fuse immunized mouse spleen
cells, by immunizing different species of animals, and by
using different routes of immunization.

Epitopes can also be directly mapped by
15   assessing the ability of different mAbs to compete with
each other for binding to the LT-ß-R using BIAcore
chromatographic techniques (Pharmacia BIAtechnology
Handbook, "Epitope Mapping", Section 6.3.2, (May 1994);
see also Johne et al., J. Immunol. Methods, 160, pp.
20   191-8 (1993)).

Individual LT-ß-R mAbs can be grouped into at
least four classes according to their ability to
cooperate in combination with other LT-ß-R mAbs in
killing tumor cells in cytolytic assays (Example 8; Table
25   1). For example, the BDA8 mAb in Group I does not act in
combination with the AGH1 mAb in Group I to promote tumor
cell cytotoxicity. Likewise, the Group III BKA11 and
CDH10 mAbs do not cooperate in a tumor cell cytotoxicity
assay.

30   Figure 5A-C shows the effects of administering
representative anti-LT-ß-R mAbs alone and in pairwise
combination to tumor cells in cytotoxicity assays in the
presence of IFN-γ as a LT-ß-R activating agent. The
Group IV anti-LT-ß-R mAb CBE11 used alone has a slight
35   cytotoxic effect which is enhanced in combination with

the Group II mAb BHA10 (**Figure 5A**). CBE11 elicits a similar effect with the Group III mAb CDH10 (**Figure 5B**).

The cytotoxicity caused by administering a combination of anti-LT-ß-R mAbs in solution is not
5   peculiar to the HT29 tumor cell line. **Figure 5C** shows that the Group I AGH1 mAb and the Group III CDH10 mAb act synergistically in killing two different tumor cell lines (HT29 cells and WiDr cells) derived from human adenocarcinoma tumors.

10   **Summary of Anti-LT-ß-R mAbs Characteristics**

All of the anti-LT-ß-R mAbs of this invention, when cross-linked by immobilization, act as LT-ß-R activating agents in the presence of a second LT-ß-R activating agent such as IFN-γ. The ability of anti-LT-
15   ß-R mAbs to act as LT-ß-R activating agents in the presence or absence of LT-α1/ß2 in solution often varies according to the state of the cells at the time of the test. Table 2, infra, summarizes the properties of the anti-LT-ß-R mAbs characterized by this invention.

20   The Group I mAbs BDA8 and AGH1 do not function as LT-ß-R activating agents in solution with LT-α1/ß2. The BDA8 mAb actually blocks the anti-tumor effect of LT-α1/ß2 (**Figure 3B** and Table 2). In contrast, the Group II anti-LT-ß-R mAbs BCG6 and BHA10 have mixed agonistic
25   and antagonistic effects when administered with LT-α1/ß2. The Group III anti-LT-ß-R mAbs BKA11 and CDH10 are unique in their ability to act as LT-ß-R activating agents which potentiate anti-tumor effects in the presence of LT-α1/ß2 and a second LT-ß-R activating agent such as IFN-γ
30   without exhibiting the antagonistic effects often seen with the Group II mAbs BCG6 and BHA10.

It is important to keep in mind that the classification of the anti-LT-ß-R mAbs based on their ability to cooperate in tumor cell cytolytic assays

reflects that they interact with distinct epitopes of the
LT-ß-R. The mAbs comprising a single group do not,
however, necessarily have the same binding affinities for
their cognate epitopes. Thus the variable results seen
5 when comparing the effects of different mAbs belonging to
the same or different groups may represent differences in
binding affinities. Accordingly, it is possible that a
Group I or a Group IV mAb with a higher binding affinity
for the LT-ß-R could be isolated which would function
10 like the Group III mAbs as a LT-ß-R activating agent in
the presence of LT-α1/ß2.

The hybridoma cell lines or subclones thereof
which produce the anti-LT-ß-R mAbs described above were
deposited on January 12, 1995 with the American Type
15 Culture Collection (ATCC) (Rockville, MD) according to
the provisions of the Budapest Treaty, and were assigned
the ATCC accession numbers designated as follows:

	CELL LINE	mAb Name	ATCC Accession No.
a)	AG.H1.5.1	AGH1	HB 11796
b)	BD.A8.AB9	BDA8	HB 11798
c)	BC.G6.AF5	BCG6	HB 11794
d)	BH.A10	BHA10	HB 11795
e)	BK.A11.AC10	BKA11	HB 11799
f)	CB.E11.1	CBE11	HB 11793
g)	CD.H10.1	CDH10	HB 11797

All restrictions on the availability to the public of the
above ATCC deposits will be irrevocably removed upon the
granting of a patent on this application.

30 **Anti-LT-ß-R IgM Monoclonal Antibodies Function as
LT-ß-R Activating Agents**

Anti-LT-ß-R mAbs which comprise more than the
usual two IgG antigen binding sites will also function in
solution as cell surface LT-ß-R cross-linking agents, and
35 will accordingly fall within the definition of a LT-ß-R

activating agent according to this invention.   The
antigen binding sites of an anti-LT-ß-R mAb can be built
into IgM molecules -- which have ten antigen binding
sites -- using standard recombinant DNA and hybridoma
5   techniques (Example 12).

Alternatively, one may collect and enrich for
complete mouse (or other animal) IgM molecules isolated
by hybridoma fusion techniques after a single
immunization with antigen.   One way to enrich for IgM
10   molecules would be to immunize CD40 signaling-deficient
mice (Kawabe et al., _Immunity_, 1, pp. 167-78 (1994); Xu
et al., _Immunity_, 1, pp. 423-31 (1994)).   These mice
cannot effectively produce IgGs and therefore their
response to challenge by antigen is enriched for IgM
15   isotypes.

Anti-LT-ß-R IgM antibodies, by virtue of their
increased valency, can effectively aggregate LT-ß-R
molecules within the plane of the membrane, thereby
enhancing LT-ß-R signaling as compared to their IgG
20   counterparts having two antigen binding sites.   A
dramatic example of the increased efficiency of
multivalent antibodies in receptor clustering is seen
with antibodies to the Fas receptor, where the IgM form
is very potent and normal bivalent IgGs are not effective
25   in solution (Yonihara and Yonihara, _J. Exp. Med._, 169,
pp. 1747-56 (1989); Alderson et al., _Int. Immunol._, 6,
pp. 1799-1806 (1994)).

Likewise, the apo-1 mAb to the Fas receptor is
an IgG3 mAb.   This mAb is a potent cytotoxic agent which
30   relies on Fc interactions unique to IgG3 subtypes to
aggregate into larger polyvalent forms.   Removal of the
Fc region creates a $F(ab)_2$ form that cannot associate into
larger aggregates and which is inactive (Dhein et al., _J.
Immunol._, 149, pp. 3166-73 (1992)).   Thus by analogy, it

is predicted that IgM versions of anti-LT-ß-R mAbs will be potent anti-tumor agents.

Anti-LT-ß-R mAbs Inhibit Tumor Growth in Mice

The ability of a LT-ß-R activating agent such as an anti-LT-ß-R mAb to inhibit human tumor cell growth _in vitro_ (Examples 6-8 and 13) may be indicative of _in vivo_ anti-tumorigenic activity. Experiments performed in immunodeficient (SCID) mice demonstrate that an anti-LT-ß-R mAb (CBE11) can efficiently block tumor formation by human adenocarcinoma WiDr cells (Example 14; **Figure 6**). Mice inoculated subcutaneously (s.c.) with WiDr cells form measurable tumors within two weeks. When mice were treated i.p. with the CBE11 mAb at the same time as the WiDr cells were inoculated s.c., tumor outgrowth was dramatically blocked (**Figure 6A**). The anti-tumor action of the CBE11 anti-LT-ß-R mAb was enhanced by adding IFN-γ; CBE11 was effective, however, even without exogenous IFN-γ. In the CBE11 + IFN-γ group, 7 of 16 animals completely lacked tumors, whereas the remaining animals had small nodules that had not progressed at 2 months. The CBE11 alone treated mice were similar to the CBE11 + IFN-γ group at 30 days. The CBE11 alone treated mice, however, eventually developed slowly growing tumors. There were statistically significant differences between the CBE11 (+/- IFN-γ) groups and the control groups (saline, IFN-γ alone and control anti-human LFA-3 mAb (1E6) + IFN-γ), but no significant differences among the control groups. The 1E6 and CBE11 mAbs are both IgG1 antibodies. The 1E6 mAb effectively coats the tumor cells but does not block tumor growth. Thus complement or natural killer cell-mediated events are not the sole basis for the anti-tumor activity of the CBE11 anti-LT-ß-R mAb .

The efficacy of the CBE11 mAb in inhibiting
tumor growth _in vivo_ in the absence of exogenous IFN-γ
was unexpected since there was a dependence on IFN-γ for
measurable LT-ß-R-based _in vitro_ cytotoxic effects.
5 Either there is some crossover of mouse IFN-γ onto human
IFN-γ receptors, or other mechanisms may be involved _in
vivo_.

The CBE11 anti-LT-ß-R mAb can also inhibit
growth of an established tumor in mice (**Figure 6B**). Mice
10 were inoculated s.c. with WiDr human adenocarcinoma cells
at day 1 and tumors were allowed to develop for 15 days
(Example 14). Tumors in animals treated i.p. with IFN-γ
alone, or with the control anti-human LFA-3 mAb (1E6) +
IFN-γ, continued to increase in size over the course of
15 the 7-week experiment. In contrast, tumors treated with
the CBE11 anti-LT-ß-R mAb (+ IFN-γ or alone) stopped
growing, and following three injections of CBE11 antibody
over a three week period, tumor growth was arrested out
to 49 days post-inoculum when the experiment was
20 terminated (**Figure 6B**).

These experiments demonstrate that an anti-
LT-ß-R mAb which activates LT-ß-R signaling can
effectively inhibit tumor formation at early stages and
can also block continued tumor cell growth at later
25 stages of tumorigenesis _in vivo_. These experiments also
demonstrate that administration of a single LT-ß-R
activating agent may be effective for treating or
reducing the advancement, severity or effects of
neoplasia in an affected animal.
30 The procedures described in Example 14 may be
used to identify LT-ß-R activating agents according to
this invention which function alone or in combination to
inhibit tumor cell growth _in vivo_. It is envisioned that
other LT-ß-R activating agents -- including but not
35 limited to those identified using _in vitro_ tumor cell

cytotoxicity assays -- may have similar anti-tumor
effects in vivo when administered either alone or in
combination to animals or humans.

The Use of IFN-γ and Other LT-ß-R Activating Agents

5   The cytotoxic effects of LT-α/ß heteromeric
complexes and of cross-linked or multiple anti-LT-ß-R Abs
on tumor cells is enhanced by the presence of a
LT-ß-R activating agent, particularly IFN-γ. Human
adenocarcinoma cells of intestinal origin (HT29 cells)
10   have previously been shown to be sensitive to FasR
signaling (Yonehara and Yonehara, J. Exp. Med., 169, pp.
1747-56 (1989)); and to TNF and LT-α in the presence of
IFN-γ (Browning et al., J. Immunol., 143, pp. 1859-67
(1989)).

15   The amount of LT-ß-R activating agent required
to enhance the anti-tumor activity of LT-α/ß heteromeric
complexes, anti-LT-ß-R Abs or other LT-ß-R activating
agents of this invention will depend on the cell or
tissue type being treated, and also with the mode of
20   treatment, and can be determined empirically using
routine procedures. The LT-ß-R activating agent can be
provided at a concentration or delivered at a rate
determined to be effective in conjunction with other
LT-ß-R activating agents administered, taking into
25   consideration the factors listed above.

               Alternatively, endogenous LT-ß-R activating
agents such as interferons like IFN-γ, which may be
produced by cells or tissue surrounding the target tumor
cells, can be relied upon. Endogenous IFN-γ is normally
30   produced upon viral infection, and is also found in the
vicinity of tumors (Dinge et al., Immunity, 1, pp. 447-56
(1994)).

               Any agent which is capable of inducing
interferons, preferably IFN-γ, and which potentiates the

cytotoxic effects of LT-α/ß heteromeric complexes and
anti-LT-ß-R mAbs on tumor cells falls within the group of
LT-ß-R activating agents of this invention. While virus
infection normally induces IFN-γ production, the levels

5   of endogenous IFN-γ may be enhanced by other agents
(Example 10). For example, clinical experiments have
demonstrated interferon induction by double stranded RNA
(dsRNA) treatment. Accordingly, polyriboguanylic/
polyribocytidylic acid (poly-rG/rC) and other forms of

10   dsRNA are effective as interferon inducers (Juraskova et
al., Eur. J. Pharmacol., 221, pp. 107-11 (1992)).

The interferon stimulator from Glycyrrhiza
glabra (Acharya et al., Indian J. Med. Res., 98, pp. 69-
74 (1993)), and pharmaceutical agents, many of which are

15   orally administrable, may also be used to boost
endogenous interferon levels. Such interferon inducers
include: imiquimod (Bernstein et al., Antiviral Res.,
20, pp. 45-55 (1994)); saparal (Paramonova et al., Vopr.
Virusol., 39, pp. 131-34 (1994)); aryl pyrimidones such

20   as bropirimine (Onishi and Machida, Hinyokika Kiyo, 40,
pp. 195-200 (1994)); Ridostin (Cheknev et al., Vopr.
Virusol., 39, pp. 125-28 (1994)).

Several of these interferon inducing agents
have been characterized as inducers of type I interferons

25   such as IFN-α. Type I interferons can also function as
LT-ß-R activating agents but are less potent than IFN-γ.

**Treatments Using LT-α/ß Complexes and LT-ß-R Activating
Agents**
The compositions of this invention will be

30   administered at an effective dose to treat the particular
clinical condition addressed. Determination of a
preferred pharmaceutical formulation and a
therapeutically efficient dose regiment for a given
application is well within the skill of the art taking

35   into consideration, for example, the condition and weight

of the patient, the extent of desired treatment and the tolerance of the patient for the treatment.

Typically, humans can tolerate up to 100-200 $\mu g/m^2$ of TNF before serious toxicity is manifested
5 (Schiller et al., Cancer Res., 51, pp. 1651-58 (1991)). In mice, dosages in the range of 1-5 μg/mouse/day given with $5x10^4$ units of recombinant human IFN-γ caused human primary tumor regression (Balkwill et al., CIBA Foundation Symposium (1987); Havell et al., J. Exp. Med.,
10 167, pp. 1067-85 (1988)). Based on the relative effectiveness of TNF and LT-α1/ß2 in the HT29 cytolytic assays, approximately 5-25 μg/mouse/day of LT-α1/ß2 will provide a therapeutic dose range. Extrapolating to the human, it is expected that LT-α1/ß2 dosages of at least 1
15 mg/m^2 will be required in combination with an LT-ß-R activating agent such as IFN-γ.

Historically, IFN-γ therapy has been undertaken either at maximum tolerated doses in the range of 100-250 $\mu g/m^2$, or at "immunomodulatory" levels in the range of
20 10-25 $\mu g/m^2$ (see e.g. Kopp et al., J. Immunother., 13, pp. 181-90 (1993)). Combination therapies with two interferons have used $4x10^6$ units/m^2 of IFN-α and approximately 250 $\mu g/m^2$ of IFN-γ (Niederle et al., Leuk. Lymphoma, 9, pp. 111-19 (1993)). Intermediate doses of
25 about 25-100 $\mu g/m^2$ of IFN-γ in combination with the LT-α/ß heteromeric complexes or purified anti-LT-ß-R-Abs described herein are expected to be suitable starting points for optimizing treatment doses.

Administration of the LT-α/ß heteromeric
30 complexes and cross-linked anti-LT-ß-R Abs of this invention, including isolated and purified forms of the antibodies or complexes, their salts or pharmaceutically acceptable derivatives thereof, may be accomplished using any of the conventionally accepted modes of

administration of agents which exhibit anti-tumor
activity.

The pharmaceutical compositions used in these
therapies may also be in a variety of forms. These
5   include, for example, solid, semi-solid and liquid dosage
forms such as tablets, pills, powders, liquid solutions
or suspensions, suppositories, and injectable and
infusible solutions. The preferred form depends on the
intended mode of administration and therapeutic
10   application. Modes of administration may include oral,
parenteral, subcutaneous, intravenous, intralesional or
topical administration.

The LT-α/ß heteromeric complexes, IFN-γ, and
anti-LT-ß-R Abs may, for example, be placed into sterile,
15   isotonic formulations with or without cofactors which
stimulate uptake or stability. The formulation is
preferably liquid, or may be lyophilized powder. For
example, the LT complexes and/or anti-LT-ß-R Abs and IFN-
γ may be diluted with a formulation buffer comprising 5.0
20   mg/ml citric acid monohydrate, 2.7 mg/ml trisodium
citrate, 41 mg/ml mannitol, 1 mg/ml glycine and 1 mg/ml
polysorbate 20. This solution can be lyophilized, stored
under refrigeration and reconstituted prior to
administration with sterile Water-For-Injection (USP).
25   The compositions also will preferably include
conventional pharmaceutically acceptable carriers well
known in the art (see for example Remington's
Pharmaceutical Sciences, 16th Edition, 1980, Mac
Publishing Company). Such pharmaceutically acceptable
30   carriers may include other medicinal agents, carriers,
genetic carriers, adjuvants, excipients, etc., such as
human serum albumin or plasma preparations. The
compositions are preferably in the form of a unit dose
and will usually be administered one or more times a day.

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The pharmaceutical compositions of this invention may also be administered using microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in, near, or
5   otherwise in communication with affected tissues or the bloodstream. Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shaped articles such as suppositories or microcapsules. Implantable or microcapsular sustained
10   release matrices include polylactides (U.S. Patent No. 3,773,319; EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22, pp. 547-56 (1985)); poly(2-hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al., J. Biomed. Mater.
15   Res., 15, pp. 167-277 (1981); Langer, Chem. Tech., 12, pp. 98-105 (1982)).

Liposomes containing LT-α/ß heteromeric complexes and/or anti-LT-ß-R Abs and IFN-γ can be prepared by well-known methods (See, e.g. DE 3,218,121;
20   Epstein et al., Proc. Natl. Acad. Sci. U.S.A., 82, pp. 3688-92 (1985); Hwang et al., Proc. Natl. Acad. Sci. U.S.A., 77, pp. 4030-34 (1980); U.S. Patent Nos. 4,485,045 and 4,544,545). Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type
25   in which the lipid content is greater than about 30 mol.% cholesterol. The proportion of cholesterol is selected to control the optimal rate of LT complex and/or anti-LT-ß-R Abs and IFN-γ release.

The LT-α/ß heteromeric complexes and anti-
30   LT-ß-R Abs of this invention may also be attached to liposomes containing other LT-ß-R activating agents, chemotherapeutic agents or IFN-γ to supplement the IFN-γ typically found in the region of tumors. Attachment of LT complexes and anti-LT-ß-R Abs to liposomes may be
35   accomplished by any known cross-linking agent such as

heterobifunctional cross-linking agents that have been
widely used to couple toxins or chemotherapeutic agents
to antibodies for targeted delivery. Conjugation to
liposomes can also be accomplished using the

5   carbohydrate-directed cross-linking reagent 4-(4-
maleimidophenyl) butyric acid hydrazide (MPBH) (Duzgunes
et al., J. Cell. Biochem. Abst. Suppl. 16E 77 (1992)).

**Advantages of therapeutic compositions based on
anti-LT-ß-R activation**

10   An anti-tumor therapy based upon LT-ß-R
activation would have several advantages. LT-ß-R binds
to LT-α/ß heteromeric complexes with high affinity in ß/ß
clefts, and with lower affinity in α/ß clefts created at
the interfaces between adjacent LT-α and LT-ß subunits.

15   In contrast, the TNF receptors bind to LT-α/ß heteromeric
complexes with high affinity only in α/α clefts.
Accordingly, purified LT-α1/ß2 complexes bind with high
affinity to LT-ß-R between adjacent LT-ß subunits, but
lack α/α clefts and thus do not cross-activate signaling

20   through the TNF receptors. Thus the LT-α/ß heteromeric
complexes of this invention will not stimulate TNF-
associated inflammatory responses.
               LT-α1/ß2 administration does not activate
endothelial cell changes associated with inflammatory

25   response even at relatively high levels. For this
reason, the side effects due to activation of the
inflammatory cascades observed with TNF should not be a
problem using the pharmaceutical compositions and
treatment methods to activate the LT-ß-R.

30   Human LT-α1/ß2 binds to mouse LT-ß-R
essentially as well as to human LT-ß-R. Injection of 100
µg human LT-α1/ß2 per mouse is not lethal (Example 11),
suggesting that stimulation of LT-ß-R in the whole animal

does not have the overt toxicity seen when similar
experiments were tried with FasR or p60 TNF-R activation.

The use of specific anti-LT-ß-R monoclonal
antibodies or antibody combinations to trigger this

5   pathway in humans may have several advantages over
treatment with LT-α/ß heteromeric complexes. An anti-
receptor antibody therapy will be more selective than
treating with ligand. Moreover, recombinant forms of
anti-LT-ß-R mAbs would be easier to engineer and produce

10   in large scale than the soluble LT-α/ß heteromeric
complexes.

It is envisioned that the mAbs or LT-α/ß
heteromeric complexes would be administered to tumor-
bearing people in conjunction with a conventional anti-

15   tumor therapy (i.e. radiation and chemotherapy). A
combined treatment of LT-ß-R activation with conventional
chemotherapies may provide an extra factor of tumor
killing activity that would be more likely to clear a
patient of tumorigenic cells than when conventional anti-

20   tumor therapy is used alone.

It is further possible that this approach may
have relatively few side effects and therefore could be
given in a semi-prophylactic sense in cases of carcinomas
that may not have metastasized, or in patients from

25   families who show a genetic pre-disposition for a certain
type of cancer.

The following are examples which illustrate the
LT-α/ß heteromeric complexes and the anti-LT-ß-R mAbs of
this invention and the methods used to characterize them.

30   These examples should not be construed as limiting: the
examples are included for purposes of illustration and
the present invention is limited only by the claims.

EXAMPLE 1
Generation of Baculovirus-infected Insect Cell Supernatants Containing LT-α/ß Forms

Recombinant baculovirus encoding either full
5   length LT-α or a secreted form of LT-ß were made as
described (Crowe et al., Science, 264, pp. 707-710
(1994)). High five insect cells (Invitrogen, San Diego,
CA.) were inoculated at a density of $2x10^5$ cells/ml into
7.2 liters of SF 900-II (Gibco) media without serum. The
10   culture reached $1.8x10^6$ cells/ml 48 hours later and was
infected with 150 ml ($3x10^8$ PFU/ml) of LT-ß and 300 ml of
LT-α baculovirus stocks. Two days later, the culture was
harvested and the cell debris was removed by
centrifugation. After addition of EDTA and PMSF (1 mM
15   EDTA and 150 µM PMSF final concentration), the clarified
supernatant was concentrated 10-fold by ultrafiltration
using a S1YM10 (Amicon) spiral cartridge. The
concentrate was divided into six 120 ml portions and
aliquots were stored at -70 C prior to purification.

20   ## EXAMPLE 2

Preparation of Soluble LT-ß Receptors as Immunoglobulin Fc Chimera

The extracellular domain of LT-ß-R up to the
transmembrane region was amplified by PCR from a cDNA
25   clone using primers that incorporated NotI and SalI
restriction enzyme sites on the 5' and 3' ends,
respectively (Browning et al., J. Immunol., 154, pp. 33-
46 (1995)). The amplified product was cut with NotI and
SalI, purified and ligated into NotI-linearized vector
30   pMDR901 along with a SalI-NotI fragment encoding the Fc
region of human IgG1. The resultant vector contained the
dihydrofolate reductase gene and the LT-ß-R-Fc chimera
driven by separate promoters. The vector was
electroporated into CHO dhfr⁻ cells and methotrexate-
35   resistant clones were isolated as per standard

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procedures. The LT-ß-R-Fc is secreted into the medium
and an ELISA assay was used to select for cell lines
producing the highest level of the chimeric protein. A
high-producing cell line was grown to large numbers and

5   conditioned medium collected. The pure protein was
isolated by Protein A sepharose fast flow affinity
chromatography.

EXAMPLE 3

Affinity Purification of LT-α1/ß2
Using TNF-R and LT-ß-R

10   To prepare resins for the receptor affinity
purification of LT forms, purified preparations of
LT-ß-R-Fc (as described in Example 2 herein) and TNF-R
p60-Fc (Crowe et al., Science, 264, pp. 707-10 (1994))

15   were immobilized on CNBr-sepharose (Pharmacia) at 5 mg/ml
resin essentially following the manufacturer's
specifications. The resins were put through one elution
cycle prior to use. A portion (120 ml) of the S1Y10
concentrate was passed over two sequential p60 TNF-R-Fc

20   columns, which bind LT-α and LT-α2/ß1. The flow through,
which contained LT-α1/ß2 and LT-ß, was passed over a
LT-ß-R-Fc column. The column was washed with 5 volumes
each of PBS, PBS with 0.5 M NaCl and PBS, and then the
LT-α and LT-α2/ß1 complexes were eluted with 25 mM sodium

25   phosphate, 100 mM NaCl, pH 3.5. Elution fractions were
immediately neutralized with 1/20 volume of 0.5 M sodium
phosphate, pH 8.6 and stored on ice. Fractions
containing protein were identified by absorbance at 280
nm, peak fractions were pooled and the elution pools from

30   the columns were analyzed by SDS-PAGE stained with
coomassie brilliant blue. Elution as described above
yielded greater than 95% pure LT-α1/ß2.

EXAMPLE 4

Characterization of the Purified LT-α1/ß2 Ligands

The fractions of Example 3 were sized by gel exclusion chromatography to assess whether trimers were

5   formed and if aggregates were present. A TSK G3000 sw x2 column was used at a flow rate of 0.5 ml/min to size separate a BioRad gel filtration protein standard, and the three different LT trimers, LT-α3, LT-α2/ß1 and LTα1/ß2. **Figure 1A** shows that very little, if any of the

10   LT-α1/ß2 trimers show up as high molecular weight aggregate. Comparison to size standards shows that the three forms are all trimeric, i.e. about 50-60 kDa. Assuming the trimer, the stoichiometry of LT-α to LT-ß contained in the purified LT-α1/ß2 and LT-α2/ß1 fractions

15   was evaluated by either densitometry of the coomassie stained gels or by peak height analysis of the two peaks following resolution on C4 reverse phase HPLC. Both measurements confirmed the identity of the fractions eluted off the affinity columns as described above.

20   The purity of the preparations was further assessed by ion exchange chromatography using BioCAD instrumentation to run pH maps of LT-α1/ß2 and LT-α2/ß1 on the weak cation exchanging resin under several different buffer systems. The method that exhibited the

25   greatest ability to cleanly retain and separate the three trimers incorporated a POROS CM (carboxymethyl) column run at 5 ml/min with a 16.66 mM MES, 16.66 mM HEPES, 16.66 mM Na Acetate pH 6.5 buffer and eluting with a 1 M NaCl gradient over 20 column volumes. The BioCAD

30   chromatograms of LT-α1/ß2 and LT-α2/ß1 complexes are shown in **Figure 1B**. Each trimer, LT-α3, LT-α2/ß1 and LT-α1/ß2 eluted at a different salt concentration and there was no evidence for cross contamination of more than 1-2% in the various preparations.

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EXAMPLE 5

Killing of HT29 Human Adenocarcinoma Cells
by Soluble LT-α1/ß2 Complexes

The HT29 cytolytic assay has been previously
5   described (Browning and Ribolini, J. Immunol., 143, pp.
1859-67 (1989)). In a typical assay, serial dilutions of
LT-α1/ß2 (and other cytokines where applicable) were
prepared in 0.05 ml in 96 well plates and 5000
trypsinized HT29-14 cells added in 0.05 ml of media
10   containing 0 or 80 U/ml (anti-viral units) of human
IFN-γ. HT29-14 cells are from a subclone of the original
ATCC-derived HT29 line that is more homogeneous. HT29-14
cells were used in the assays; all of these results can
also be observed using the original ATCC-derived HT29
15   line. After 3-4 days, mitochondrial reduction of the dye
MTT was measured as follows: 10 1 of MTT was added and
after 3 hours, the reduced dye dissolved with 0.09 ml of
isopropanol with 10mM HCl, and the O.D. measured at
550 nm. Soluble receptor forms prepared as described
20   herein, or pure human IgG were added in 10 μl prior to
the cells to give a final concentration of 5 μg/ml.

Figure 2A shows the killing of HT29 cells by
treating with anti-Fas receptor mAb CH-11 (which
stimulates FasR signaling); TNF, LT-α3, LT-α1/ß2 and
25   LT-α2/ß1 ligands in conjunction with IFN-γ. Visual
inspection of the cells treated with LT-α1/ß2 reveals
that this agent kills cells rather than just blocking
cell proliferation. In the absence of IFN-γ, no effects
are observed, reflecting the unusual ability of IFN-γ to
30   influence how cells interpret signaling from the TNF
family of receptors. Interferons α and ß were 100-fold
less effective than IFN-γ as quantitated based on anti-
viral activity units.

Figure 2B shows the inhibition of LT-α1/ß2
35   killing by soluble LT-ß-R-Fc but not p60-TNF-R-Fc,

demonstrating that cytotoxicity is specific to LT-α1/ß2. The lack of inhibition by p60-TNF-R-Fc indicates that contaminating LT-α (known to be less than 1%) cannot account for the cytotoxic activity of LT-α1/ß2.

5   EXAMPLE 6

Anti-LT-ß-R mAbs Potentiate the Killing of HT29 Cells by LT-α1/ß2 Complexes

            Cytolytic assays were performed as described in Example 5, except that IFN-γ and anti-LT-ß-R mAbs (0.01 -
10   1000 ng/ml series) were added to the cells at 2x final concentration and then 50 µl of the cell solution were added to the wells containing diluted LT-α1/ß2. Growth was assessed as described in Example 5. **Figure 3** shows the differential effects of two different anti-LT-ß-R
15   mAbs in their ability to potentiate LT-α1/ß2 cytotoxic activity. **Figure 3A** shows that the anti-LT-ß-R mAb CDH10 potentiates LT-α1/ß2 cytotoxic activity in a dosage-dependent manner. **Figure 3B** shows the effects of another anti-LT-ß-R mAb, BDA8, in the same assay. The
20   BDA8 mAb inhibits the cytotoxic activity of LT-α1/ß2 rather than potentiating tumor cell death.

                             EXAMPLE 7

Immobilized Anti-LT-ß-R mAbs
Can Kill HT29 Tumor Cells

25   To immobilize anti-LT-ß-R mAbs onto a plastic surface, 96-well tissue culture plates were coated with 50 µl of 10 µg/ml goat anti-mouse Fc polyclonal antibody (Jackson ImmunoResearch), washed and blocked with 5% FCS in PBS, followed by capture of the indicated anti-
30   LT-ß-R mAb and another wash. HT29 cells were plated into the mAb-coated wells, and cytolytic assays were conducted

as in Example 5. **Figure 4A** illustrates the cytotoxic
effects of immobilized BDA8 and CDH10 anti-LT-ß-R mAbs on
HT29 cells. Each mAb individually elicits cytotoxicity
on tumor cells when it is immobilized onto a surface.

5   **Figure 4B** shows that the same BDA8 and CDH10 anti-LT-ß-R
mAbs tested individually in solution are not cytotoxic
and thus the cytolytic activity of a single anti-LT-ß-R
mAb <u>in vitro</u> appears to be a function of its
immobilization.

10   **EXAMPLE 8**

**A combination of anti-LT-ß-R mAbs in solution directed
against distinct epitopes kill HT29 cells**

Growth of HT29 cells was assessed as described
in Example 5 except that either one or two anti-LT-ß-R

15   mAbs were included in the growth medium. **Table 1** shows
the effects on HT29 cells observed when various anti-
LT-ß-R mAbs were included in solution (i.e., not
immobilized on plastic). The anti-LT-ß-R mAbs can be
arranged into groups I-IV based on their relative

20   abilities to work in combination with each other in a
HT29 cytolytic assay. The anti-LT-ß-R mAbs results
generated in cytolytic assays parallel receptor binding
data which suggest that the mAbs in each different group
recognize different epitopes of the LT-ß-R.

Table 1. Combinations of soluble anti-LT-ß-R mAbs are cytotoxic to human adenocarcinoma HT29 cells. Anti-LT-ß-R mAbs are grouped into Groups I, II, III and IV based on their effects in combination with each other in HT29 cell cytolytic assays. Pluses refer to the relative level of cytolytic effects of the mAb combination on HT29 cells in the presence of 80U/ml IFN-γ. nr = not relevant; nd = not determined.

| | | Second mAb | | | | | | |
| | | Group I | | Group II | | Group III | | Group IV |
Group	First mAb	BDA8	AGH1	BCG6	BHA10	BKA11	CDH10	CBE11
I	BDA8	nr	–	+	++	+	nd	nd
	AGH1	–	nr	++	+++	++	nd	nd
II	BCG6	++	++	nr	–	+++	nd	nd
	BHA10	++	+++	–	nr	+	+++	++++
III	BKA11	+	++	+++	nd	nr	–	nd
	CDH10	++	++	++	+++	–	nr	+++
IV	CBE11	nd	+	+	++++	nd	++++	nr

Figure 5A-D quantitates the effects of including in the HT29 cytolytic assay representative pairwise combinations of cooperating anti-LT-ß-R mAbs directed against different epitopes of LT-ß-R. **Figure 5A** shows the cytotoxic effects of BHA10 and CBE11, **Figure 5B** of CDH10 and CBE11, and **Figure 5C** of CDH10 and AGH1, alone and in combination. **Figure 5D** shows the cytotoxic effects of the CDH10 and AGH1 mAb combination in a different tumor cell line called WiDr.

     Table 2 summarizes the characteristics of the representative anti-LT-ß-R mAbs of this invention.

Table 2

Summary of Mouse Anti-human LT-β-R mAbs

mAb Group	mAb Name	Cell Staining[a]	Blocking Receptor Binding[b]	HT29 Cytotoxicity		
				mAb Immobilized on Plastic[c]	Soluble mAb alone	Soluble mAb with Ltα1β1
I	BDA8	+++	+++	+	+/−[d]	inhibits
I	AGH1	+++	+++		+/−	inhibits
II	BCG6	+++	++	+	+/−	mixed
II	BHA10	+++	+++	+	+/−	mixed
III	BKA11	+++	+/−	+	−	potentiates
III	CDH10	+++	+/−	+	+/−	potentiates
IV	CBE11	+++	+++	+	+/−	no effect
Controls						
	MOPC21	−	−	−	−	no effect
	HT29/26	−	nd	−	−	no effect
	TS 2/9[e]	nd	nd	−	−	no effect

[a]FACS staining of CHO cells transfected with the LT-β-R.
[b]Assay assessed whether antibody blocks binding of soluble receptor to the activated T-cell hybridoma II-23. nd = not done.
[c]HT-29 cells were grown with IFN-γ on anti-LT-β-R coated plates as defined in the methods.
[d]Variable, partial inhibition in some assays, no effects in others.
[e]Anti-human LFA-3, a mouse IgG1.

EXAMPLE 9

Reliance on Endogenous IFN-γ
For the Treatment of Tumor Cells

IFN-γ, a preferred LT-ß-R activating agent of
the present invention, is a cytokine which exhibits anti-
tumor activity and which is tolerated in humans.
Endogenous IFN-γ present in the environment surrounding a
tumor may be at sufficiently high concentrations to
function as a LT-ß-R activating agent of this invention
without adding exogenous IFN-γ. The concentration of
IFN-γ in the vicinity of a tumor may be ascertained using
standard immunochemical techniques with tissue samples
from the region of the tumor. If the endogenous
concentration of IFN-γ is high enough to elicit anti-
tumor activity in combination with the LT-α/ß heteromeric
complexes or anti-LT-ß-R mAbs of this invention (as
determined by the cytolytic assays described herein),
then IFN-γ need not be administered as a second LT-ß-R
activating agent in the compositions or methods of this
invention.

EXAMPLE 10

Induction of Endogenous IFN-γ As A LT-ß-R
Activating Agent For the Treatment of Tumor Cells

Compounds which can induce the endogenous
production of interferons such as IFN-γ fall within the
group of LT-ß-R activating agents of this invention. For
example, interferons can be induced by treating with
double-stranded RNA molecules such as polyribo-
guanylic/polyribocytidylic acid (poly-G/C).

Female C57/b16 (6-8 weeks old) can be injected
with 18 mg (600 mg/kg) of D-galactosamine which
sensitizes mice to the effects of TNF and other anti-
tumor agents. A series of concentrations of poly-G/C
(Juraskova et al., Eur. J. Pharmacol., 221, pp. 107-11

(1992)) in a neutral saline solution is added to purified LT-α1/β2 (10-100µg) and the solution administered to mice as an intraperitoneal (i.p.) injection. The anti-tumor activity of LT-α1/β2 will be enhanced by the presence of

5   poly-rG/rC double stranded RNA.

Similarly, the interferon stimulator from the plant Glycyrrhiza glabra (Acharya et al., Indian J. Med. Res., 98, pp. 69-74 (1993)) may be administered to humans intravenously at doses ranging from 40-100 ml/day. The

10   optimal dose for LT-β-R activation in the presence of either LT-α/β heteromeric complexes or anti-LT-β-R Abs may be determined empirically and will depend on factors such as the tumor type, mode of delivery and delivery schedule.

15   Imiquimod R-837 (Bernstein et al., Antiviral Res., 20, pp. 45-55 (1994)); Saparal (Paramonova et al., Vopr. Virusol., 39, pp. 131-34 (1994)); Bropirimine (Onishi and Machida, Hinyokika Kiyo, 40, pp. 195-200 (1994)); or Ridostin (Cheknev et al., Vopr. Virusol., 39,

20   pp. 125-28 (1994)), may also be administered as LT-β-R activating agents in conjunction with LT-α/β heteromeric complexes, anti-LT-β-R Abs, or a combination thereof. In each case, the preferred modes of delivery and optimal doses can be determined empirically using the published

25   reports as starting points for optimization by routine clinical procedures.

EXAMPLE 11

Mice Tolerate Injections of Human LT-α1/β2

Female C57/b16 (6-8 weeks old) acclimated to

30   the facility for several days were injected i.p. with 18 mg (600 mg/kg) of D-galactosamine, which sensitizes mice to the effects of TNF and other anti-tumor agents. Either human TNF, LT-α or LT-α1/β2 was then given i.p.

Table 3 documents the survival of treated mice 24 hours after injection.

Table 3

Agent	Dose (µg/animal)	Survival
saline	–	4/4
hu-TNF	0.2	0/6
hu-TNF	1.0	0/2
hu-TNF	10	0/4
hu-LT-α	0.2	2/2
hu-LT-α	1.0	2/2
hu-LT-α1/ß2	10	2/2
hu-LT-α1/ß2	100	2/2

EXAMPLE 12

Construction of a recombinant anti-LT-ß-R IgM monoclonal antibody

Using the anti-tumor cytotoxicity assays described above coupled with standard tumor growth models in immunodeficient mice, an anti-LT-ß-R IgG with suitable properties can be selected. Universal primers which hybridize to each of the variable domains of the IgG heavy and light chains of the selected anti-LT-ß-R IgG can be used to prepare variable domain DNA from RNA isolated from the secreting hybridoma cell line using standard reverse transcriptase/PCR methodologies. These protocols have been described (Arulanandam et al., J. Exp. Med., 177, pp. 1439-50 (1993); Lane et al., Eur. J. Immunol., 22, pp. 2573-78 (1993); Traunecker et al., Nature, 339, pp. 68-70 (1989)).

The amplified products are then assembled into vectors containing the human CH1, CH2 and CH3 µ chain domains. Co-expression of the two chains in a single host will allow assembly of the heavy and light chains into a pentameric IgM molecule. This molecule is a

chimera composed of mouse variable regions coupled to human constant regions.

Alternatively, a process using PCR to amplify DNA encoding only the actual binding regions of the variable regions can be used. Amplified DNA is then inserted into vectors containing all of the human IgG sequences except for the actual amino acids involved in binding the antigen. Such constructs are called "humanized" antibodies and the detailed methods for their production are well-known (e.g. WO 94/04679).

EXAMPLE 13

Anti-LT-ß-R IgM Monoclonal Antibodies Function as LT-ß-R Activating Agents

Anti-LT-ß-R IgM antibodies can be prepared in a recombinant form as described in Example 12. Alternatively, complete mouse IgMs isolated by hybridoma fusion techniques using primary immunization of normal mice or extensive immunization of CD40 signaling-deficient mice (Kawabe et al., Immunity, 1, pp. 167-78 (1994); Xu et al., Immunity, 1, pp. 423-31 (1994)) can be used as a source of anti-LT-ß-R IgM mAbs.

Anti-LT-ß-R IgM mAbs will be significantly more potent as LT-ß-R activating agents than their normal bivalent IgG counterparts as measured by dose-response comparisons in the HT29 cytolytic assay in the presence of IFN-γ. The anti-LT-ß-R IgM mAbs function as LT-ß-R activating agents both when they are immobilized and when they are administered in solution. In addition, we expect that they will augment the anti-tumor activity of LT-α/ß heteromeric complexes.

EXAMPLE 14

Anti-LT-ß-R Monoclonal Antibodies Inhibit
the Growth of Human Tumor Cells in SCID Mice

5   Balb/c SCID mice (Jackson Labs, Bar Harbor, ME)
were injected with 1 x 10⁶ trypsinized and washed human
adenocarcinoma WiDr cells in a volume of 0.2 ml of PBS
subcutaneously (s.c.) onto the back of the animal.
Injected WiDr cells form tumors in the mice, and the
10   ability of an anti-LT-ß-R mAb to inhibit tumor growth was
monitored. In one set of experiments, mice were treated
with or without the CBE11 anti-LT-ß-R mAb -- either with
or without human IFN-γ (10⁶ antiviral units/mouse) -- at
the same time as the WiDr cells were inoculated s.c.
15   (**Figure 6A**). Antibodies and IFN-γ were administered
alone or together by i.p. injection in 0.2 ml. Control
mice were injected with saline alone, IFN-γ alone, or a
control anti-human LFA-3 mAb (1E6) with IFN-γ. The size
of each resulting tumor was scored 30 days after
20   inoculation. Tumor volume (in cc) was calculated from
the radius as determined by caliper measurements in two
dimensions. Animals treated with CBE11 or 1E6 mAbs
received 10 µg/mouse or 50 µg/mouse of antibody (**Figure
6A;** circles with dots and open circles, respectively).

25   In another set of experiments, mice were
inoculated s.c. with the WiDr cells and tumors were
allowed to grow for 15 days before the mice were treated
with the CBE11 anti-LT-ß-R mAb (**Figure 6B**). At day 15
(before antibody treatment), the average tumor volume was
30   0.076 cc with an average diameter of 0.53 cm. The CBE11
anti-LT-ß-R mAb (50 µg) was then administered -- either
with or without human IFN-γ (10⁶ antiviral units/mouse) --
by i.p. injection in 0.2 ml to a group of 12 animals.
Injections were repeated three more times over a three
35   week period. Control groups (12 mice/group) were
injected with IFN-γ alone (10⁶ antiviral units/mouse) or

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with 50 μg of a control anti-human LFA-3 mAb (1E6) +
IFN-γ (10^6 antiviral units/mouse). The growth of the
tumors present at day 15 was scored over time, from 15 to
49 days post-tumor cell inoculation. The results shown
5   in **Figure 6B** were determined in a blinded format. Tumors
treated with CBE11 mAb either with or without IFN-γ
stopped growing. Following three injections of CBE11 mAb
(+/- IFN-γ) over three weeks, tumor growth was arrested
for at least 7 weeks post-inoculum, at which time the
10   experiment was terminated.